

## Morphology of an *Escherichia coli* Mutant with a Temperature-Dependent Round Cell Shape

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Mutants of *Escherichia coli* capable of growing in the presence of 10  $\mu$ g of mecillinam per ml were selected after intensive mutagenesis. Of these mutants, 1.4% formed normal, rod-shaped cells at 30°C but grew as spherical cells at 42°C. The phenotype of one of these *rod*(Ts) mutants was 88% cotransducible with *lip* (14.3 min), and all *lip*<sup>+</sup> *rod*(Ts) transductants of a *lip* recipient had the following characteristics: (i) growth was relatively sensitive to mecillinam at 30°C but relatively resistant to mecillinam at 42°C; (ii) penicillin-binding protein 2 was present in membranes of cells grown at 30°C in reduced amounts and was undetectable in the membranes of cells grown at 42°C. The mecillinam resistance, penicillin-binding protein 2 defect, and *rod* phenotypes all cotransduced with *lip* with high frequency. Thus the mutation [*rodA*(Ts)] is most likely in the gene for penicillin-binding protein 2 and causes the organism to grow as a sphere at 42°C, although it grows with normal rodlike morphology at 30°C. At 42°C, cells of this strain were round with many wrinkles on their surfaces, as revealed by scanning electron microscopy. In these round cells, chromosomes were dispersed or distributed peripherally, in contrast to normal rod-shaped cells which had centrally located, more condensed chromosomes. The round cells divided asymmetrically on solid agar, and it seemed that the plane of each successive division was perpendicular to the preceding one. On temperature shift-down in liquid medium many cells with abnormal morphology appeared before normal rod-shaped cells developed. Few abnormal cells were seen when cells were placed on solid medium during temperature shift-down. These pleiotropic effects are presumably caused by one or more mutations in the *rodA* gene.

Relatively inflexible bacteria have two principal forms: spherical and rod shaped. *Escherichia coli* is normally rod shaped, but there are several reports of mutants with spherical shape (2, 10, 17, 21, 22, 33). When envelopes of *E. coli* are extracted with hot sodium dodecyl sulfate, leaving the peptidoglycan component of the wall substituted only by covalently linked lipoprotein, they retain the shape of the cell from which they were derived (10, 11). The peptidoglycan is apparently the principal shape-maintaining component of the *E. coli* envelopes, but we have little knowledge as to how the shape of this component, or of the similar component in any other bacterium, is determined (9, 24). A role for outer membrane proteins in shape maintenance and determination has been invoked (11), but mutants defective in multiple major components of these proteins are morphologically normal (9).

Most round mutants of *E. coli* differ from their parent in antibiotic susceptibility, although they have been isolated by screening for a variety of phenotypes, including conditional lysis and differential sensitivity to UV or drugs (2, 21, 22, 33), many of which may be secondary consequences of alterations in the wall. Matsushashi et al. (21) reported finding a high proportion of round mutants of *E. coli* among strains selected for resistance to mecillinam after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). We have previously mapped some of these mutants by P1 transduction and have found them to be identical with or closely linked either to *rodA* or to *envB* (12). The *rodA* locus is closely linked to *lip* (12, 22). In this paper we will describe one mutant, strain R3-1, which shows a temperature-dependent change in morphology. This mutant maps at or near *rodA* and loses the characteristic rod shape of *E. coli* on growth at high temperature. It is mecillinam

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resistant and lacks penicillin-binding protein 2 (PBP2) at high temperature, suggesting that the *rodA* locus, responsible for round cell shape, is the gene for PBP2, which is a target of mecillinam action (31). We also describe studies of morphological changes in this mutant. At high temperature the nucleoid of the cell, normally seen in a relatively condensed form in thin-section electron micrographs, became dispersed. Scanning electron microscopy showed that the spherical cell has a wrinkled surface. When shifted down from 42 to 30°C in liquid medium, the cell regained its normal rod morphology after passing through a transient series of abnormal cell shapes. Light and electron microscopic data are presented which describe rod-to-sphere and sphere-to-rod transitions in this mutant.

### MATERIALS AND METHODS

**Organisms.** Bacterial strains are described in Table 1. Strains AT2538, AT1325lip9, and AB2834 were obtained from the *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn. Generalized transducing phage P1*vir*-1 was a gift from Hideo Ikeda. R3 was isolated as a mecillinam-resistant, temperature-dependent morphological mutant (rod at 30°C and round at 42°C) of AT2538 after heavy mutagenesis with NTG as described in the following section. R3-1 is a *lip*<sup>+</sup> transductant of R3 into AT1325lip9 and shows the same temperature-dependent morphological change as R3. R3-5 is a *lip*<sup>+</sup> transductant of R3 into AT1325lip9 and is rod shaped both at 30 and at 42°C.

**Media.** DYABT and M9 media have previously been described (12). DYABT*lip* is DYABT medium plus 5 ng of lipoic acid (DL-6,8-thioctic acid; Sigma Chemical Co.) per ml. P1 suspension medium is 0.05 M tris(hydroxymethyl)aminomethane (pH 7.4) at 25°C containing 1 mM MgSO<sub>4</sub> and 0.1 M NaCl. <sup>14</sup>C-labeled penicillin G (54 mCi/mmol) was purchased from Amersham/Searle Corp.

**Isolation of mutants with temperature-dependent round morphology.** *E. coli* AT2538 cells were treated with NTG (400 µg/ml) at 30°C (1, 13) for 180 min (survival  $1.7 \times 10^{-3}$ ), washed, and grown in fresh DYABT medium at 30°C. After cells reached log growth phase, they were again treated with NTG for 60 min (survival  $4.5 \times 10^{-2}$ ) as above. After these three cycles of NTG treatment, cells were suspended in 25% glycerol solution, rapidly frozen in acetone-dry ice, and stored at -70°C. These heavily mutagenized cells were spread on DYABT plates with or without mecillinam at 10 µg/ml, and plates were incubated at 30°C for 2 to 3 days. About 5% of all colonies on DYABT plates without mecillinam turned out to be mecillinam resistant. The colonies on DYABT plates containing mecillinam were first cloned on DYABT plates with mecillinam at 30°C, and then, after incubation for a few days, a single colony was picked and cloned on duplicate DYABT plates without mecillinam at 30 or 42°C. After a few days, the morphology of cells was examined by phase-contrast microscopy. A majority showed normal rod-shaped cells at 30 and 42°C. Among 834 mecillinam-resistant strains examined, we found 42 with round cells at both 30 and 42°C (*rod*), 12 with rod-shaped cells at 30°C and round cells at 42°C [*rod*(Ts)], and a single mutant with round cells at 30°C and rod-shaped cells at 42°C. Of the 834 strains, 125 showed temperature-sensitive growth. We have chosen one of the *rod*(Ts) mutants (rod at 30°C and round at 42°C), strain R3, for further study.

**Preparation of P1*vir*-1 transducing lysate.** Five milliliters of a log-phase culture of R3 was mixed with 0.05 ml of 0.1 M CaCl<sub>2</sub> and 0.01 ml of P1*vir*-1 ( $4 \times 10^8$ /ml) and incubated at 30°C for 20 min. To 0.2 ml of this mixture was added 2.5 ml of 0.65% agar in DYABT containing CaCl<sub>2</sub> (1 mM) at 51°C, and this mixture was poured onto DYABT plates (1.5% of agar) containing lipoic acid (5 ng/ml). After incubation at 30°C for 7 h to overnight, 3 ml of P1 suspension medium was added to the plate, and the top layer was scraped into a test tube. Several drops of CHCl<sub>3</sub> were added, and the mixture was blended in a Vortex mixer for a few seconds. The agar was spun down by centrifugation at low speed. A few drops of CHCl<sub>3</sub> were added

TABLE 1. Bacterial strains

Strain	Genotype and characteristics	Reference
AT1325lip9	<i>thi-1 his-4 purB15 proA2 mtl-1 xyl-5 galK2 lacY1</i> λ <sup>-</sup> <i>lip-9 str-35 supE44?</i>	12
AT2538	<i>thi-1 pyrE60 argE3 his-4 proA2 thr-1 leu-6 mtl-1 xyl-5 ara-14 galK2 lacY1 str-31</i> λ <sup>-</sup> <i>supE44?</i>	12
AB2834	<i>aroE353 mal-352 tsx-352</i> λ <sup>-</sup> λ <sup>-</sup> <i>supE42?</i>	12
R3	<i>rodA</i> (Ts); derivative of AT2538, rod at 30°C and round at 42°C	This paper
R3-1	<i>lip</i> <sup>+</sup> transductant of R3 into AT1325lip9; <i>rodA</i> (Ts)	This paper
R3-5	<i>lip</i> <sup>+</sup> transductant of R3 into AT1325lip9; <i>rodA</i> <sup>+</sup>	This paper
HL103	derivative of H2143; <i>rodA</i> (or <i>rodX</i> )	12, 21
HL18	derivative of H2143; <i>envB</i> (or <i>rodY</i> )	12, 21
AR6	<i>aroE</i> <sup>+</sup> transductant of HL18 into AB2834; <i>envB</i>	12; this paper
CS1	<i>argG</i> <sup>+</sup> transductant of HL18 into CSH57; <i>envB</i>	12; this paper
CSH57	<i>ara leu lacY purE gal trp his argG malA rpsL xyl mtl ilv metA</i> or - <i>B thi</i>	12
H2143	<i>lys dap thi</i>	12, 21
SP6 (or B6)	Mecillinam resistant and round	30

to the supernatant and mixed in the Vortex mixer for a few seconds. The clear supernatant was used as the P1 stock. The titer on AT1325lip9 was usually several times  $10^8$  per ml.

**Transduction.** Strain AT1325lip9 was grown in DYABT plus lipoic acid at 30°C. Cells (10 ml) were harvested in midlog phase and suspended in 0.5 ml of M9 medium containing 2 mM  $\text{CaCl}_2$ . A 0.1-ml sample of this suspension was mixed with 0.1 ml of a P1vir-1 phage preparation and incubated at 36°C for 15 min. M9 medium was then added (5 ml), and the cells were spun down and suspended in 0.1 ml of M9 medium. A 0.05-ml sample of this suspension was plated on medium lacking lipoic acid. After 2 days at 30°C, isolated colonies were cloned on the same medium. Two *lip*<sup>+</sup> transductants, R3-1 and R3-5, were selected for further study.

**Growth of strain R3-1 in liquid medium.** Cells were grown overnight at 30°C in DYABTlip and subcultured in the same medium at 30°C. Growth was monitored in a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc.) with a red filter (660 nm). When the culture attained exponential growth, a second dilution (1:20) was made into fresh medium preheated to 30°C.

**Temperature shift-up experiment.** When the third culture reached midexponential growth at 30°C, 5 ml was transferred into 45 ml of fresh DYABTlip at 42°C in a shaking water bath, and further growth was monitored as before.

**Temperature shift-down experiment.** After incubation of the above culture at 42°C for 90 min, 5 ml was transferred into 45 ml of fresh DYABTlip at 30°C, and growth was monitored as before.

**Phase-contrast microscopy.** Bacterial samples were fixed in glutaraldehyde and washed in distilled water as described in the next section. A Nikon phase-contrast microscope (model L-Ke, Nippon Kogaku K.K.) with a photographic attachment was used to record observation at the same magnification of 1,000 for all pictures (objective; DLL100 H1, N.A. 1.25). A bar represents 50  $\mu\text{m}$  for all pictures. Kodak Plus-X pan film was used.

**Scanning electron microscopy.** At the appropriate times, 3 ml of culture was withdrawn, and the cells were isolated by centrifugation and suspended in 0.5 ml of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After 10 to 30 h at 4°C, samples were washed twice with 1 ml of distilled water and suspended in 0.5 ml of distilled water. The washed cells were fixed on glass cover slips (no. 2 thickness, 18-mm diameter, Fisher Scientific Co.) as follows. Cover slips were coated with 75  $\mu\text{l}$  of poly-L-lysine hydrobromide (type I-B, Sigma Chemical Co.) as a 1-mg/ml stock solution in distilled water (29). After 1 h at room temperature, the coated cover slips were washed in distilled water and placed in a divided petri dish. A portion of the cell suspension (75  $\mu\text{l}$ , about  $10^7$  cells per ml) was placed on each cover slip. The petri dish was covered and stored at 4°C overnight. The suspension on the cover slip was blotted with filter paper. The cover slips with bacterial samples were put through increasing gradations of ethyl alcohol (50, 75, 90, and 100%) at 4°C for 10 min each (16). After two

further changes of 100% ethyl alcohol at room temperature for 15 min each, the samples were put through increasing gradations of isoamyl acetate in ethanol (25, 50, 75, and 100%) at room temperature for 10 min each and then transferred to 100% isoamyl acetate (16). Samples were dried in a critical-point drying apparatus (Samdri TM PVT-3, Biodynamics Research Corp.) with liquid  $\text{CO}_2$ . The duration time of purging was 20 min. The surface of the entire cover slip was coated with gold-palladium in a Hummer Vacuum Evaporator (Technics, Inc.) for 10 min at 6 V and 5 mA. The thickness of the coating was about 50 to 60 nm, according to the instruction booklet from Technics. Samples were examined in a ETEC Auto-scan (ETEC Corp.) at 20 kV.

**Transmission electron microscopy.** Samples for transmission electron microscopy were harvested by centrifugation and prefixed in Veronal-acetate buffer (15) containing 2% glutaraldehyde. After 3 h at 4°C, processing was continued as described by Kellenberger et al. (15), employing fixation with 1% osmium tetroxide, followed by dehydration with ethanol and embedding in Spurr low-viscosity embedding media (Polysciences, Inc., Warrington, Pa.). Thin sections were cut using an LKB ultramicrotome or a Sorvall MT-2 microtome, stained with 1.0% aqueous uranyl acetate followed by Reynolds lead citrate (26), and examined in a Philips 300 electron microscope at 60 kV.

**Cell counts.** Cells were counted using a Petroff-Hausser counting chamber (C. A. Hausser & Son). Usually 32 to 64 small squares, for a total of 100 to 800 bacteria, were counted. When the bacteria showed motility, 1 min of treatment in boiling water made them nonmotile.

**Time-lapse photography.** DYABTlip medium containing 1.5% agar at 50°C was poured into a square well (20 by 20 by 1 mm) made on a slide glass. After the agar cooled, 1  $\mu\text{l}$  of bacterial culture was spread on the surface by applying a cover glass (22 mm square). This covered the whole area of the well. The edge was sealed by a transparent tape. Phase-contrast microscopy was performed in environmental rooms maintained at 30 or 37°C.

## RESULTS

**Transductional mapping of the gene for temperature-dependent morphology.** Since genes controlling morphology in *E. coli* have been mapped at about 14 min (*rodX* or *rodA*) and at about 70 min (*rodY* or *envB*) (12), we transduced for selectable markers at these loci and found that the *rod(Ts)* gene of strain R3 was 88% cotransducible with *lip* (14.3 min) (Table 2). The *rodX* (or *rodA*) gene is also cotransducible with *lip* at 83 to 91% (12). The round morphology gene of SP6 was also cotransducible with *lip* with a high frequency (Table 2).

**Mecillinam sensitivity and penicillin-binding components at 30 and 42°C.** The recipient for transduction, strain AT1325lip9, was highly sensitive to mecillinam when grown at 33°C in liquid DYABT medium containing

lipoic acid (Fig. 1a). The *lip*<sup>+</sup> *rod*(Ts) transducent, R3-1, behaved similarly at 33°C, showing even greater sensitivity of growth (Fig. 1b). With both strains, maximal effect was seen at 1 μg of mecillinam per ml.

At 42°C, growth of strain AT1325lip9 was almost unaffected by 1 μg of mecillinam per ml, though it was strongly retarded by 10 μg of mecillinam per ml (Fig. 1a). Strain R3-1 differed from AT1325lip9 only in showing almost complete resistance to even 10 μg of mecillinam per

ml at 42°C (Fig. 1b).

Strain R3, the donor for transduction, differed from R3-1 in being highly resistant to mecillinam even at 30°C both in liquid medium and on solid medium, although growth of this strain at 30°C was slowed in the presence of the drug (data not shown).

R3, the *rod*(Ts) transducent R3-1, and the other randomly chosen *rod*(Ts) transductants all lacked PBP2 at 42°C (Fig. 2 and unpublished data). Strain AT1325lip9 had the normal complement of six PBPs at both 30 and 42°C (data not shown). A *lip*<sup>+</sup> transducent, R3-5, for which R3 was the donor and which was rod shaped at both 30 and 42°C, also showed the normal six PBPs at both 30 and 42°C (Fig. 2). Prolonged autoradiographic exposure revealed a very small amount of PBP2 in membrane fractions prepared from 30°C cultures of strain R3 and R3-1 (Fig. 2 and unpublished data). Strain SP6 lacks PBP2, and round *lip*<sup>+</sup> transductants of AT1325lip9, using SP6 as donor, also had no detectable PBP2, whereas *lip*<sup>+</sup> *rod*<sup>+</sup> transductants showed the normal amount of PBP2 (data not shown).

TABLE 2. Cotransduction frequency between round morphology gene and *lip*

Recipient	Selection	Donor (no. of round cells/total recipient cells selected)	
		R3 <sup>a</sup>	SP6
AT1325lip9	<i>lip</i> <sup>+</sup>	21/24	8/10

<sup>a</sup> The morphology of a total of 24 *lip*<sup>+</sup> transductants from three plates was examined. Since five *lip*<sup>+</sup> revertants were seen on three control plates, there could be as many as five revertants among the 24 *lip*<sup>+</sup> transductants examined.

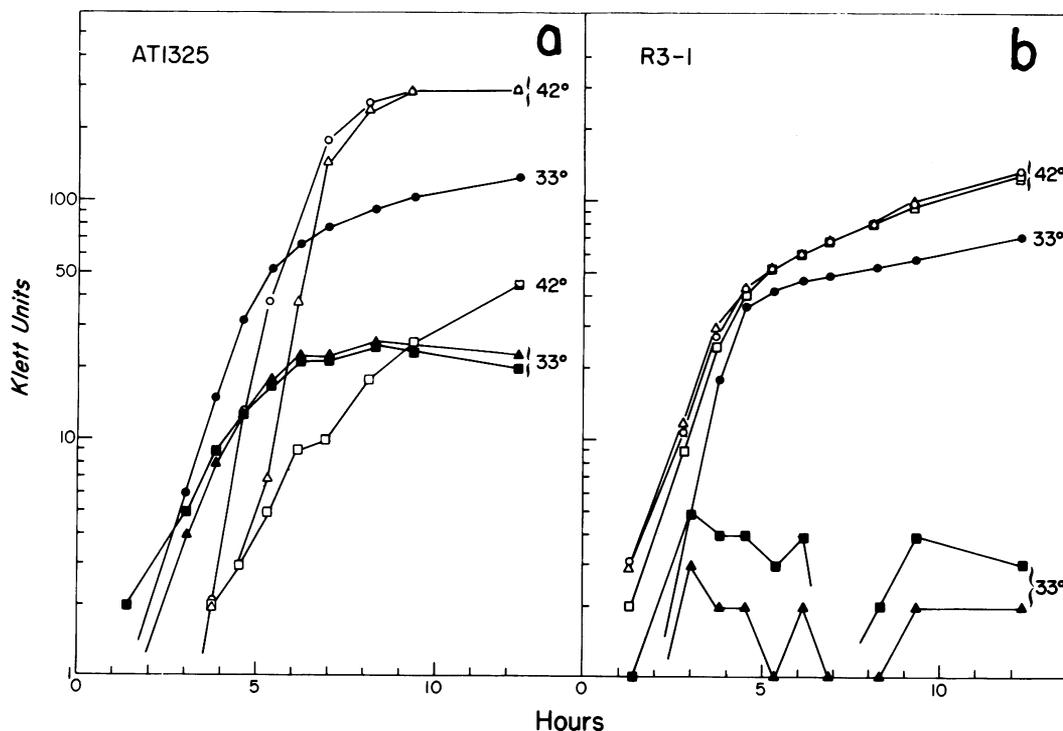


FIG. 1. Mecillinam sensitivity in liquid medium. A 0.03-ml sample of an overnight culture at 30 or 42°C was inoculated into 10 ml of DYABT medium with or without mecillinam. Growth of AT1325lip9 (a) or R3-1 (b) was followed by optical density as described in the text. Symbols: (●, ○) without mecillinam; (▲, △) 1 μg of mecillinam per ml; (■, □) 10 μg of mecillinam per ml. Open symbols show the growth at 42°C and the closed symbols, 33°C.

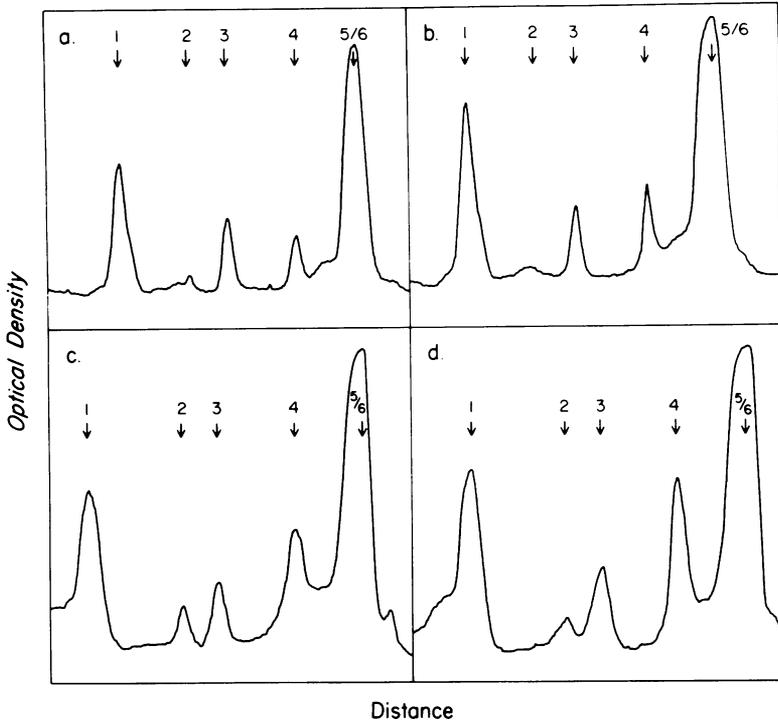


FIG. 2.  $^{14}\text{C}$ -labeled penicillin-binding proteins at 30 and 42°C. Membrane fractions were prepared from 1 liter of culture grown at 30 or 42°C.  $^{14}\text{C}$ -labeled penicillin G binding at 29°C and fluorography were done according to Spratt (30). Films were scanned in a densitometer (Ortec model 4310) in an optical density range of 0 to 3. PBPs are marked as 1 to 6. (a, b) and (c, d) are two separate experiments. Exposure time for fluorography was 6 months. (a) R3-1 grown at 30°C; (b) R3-1 at 42°C; (c) R3-5 at 30°C; (d) R3-5 at 42°C.

**Growth of strain R3-1 at 30°C and its reversible change of morphology during temperature shifts.** Strain R3-1 grew well on DYABT plates at 30°C and exhibited normal rod morphology by phase-contrast microscopy. At 42°C it formed slightly smaller colonies, and the cells were spherical and of variable size. Figure 3 shows the growth of R3-1 in liquid culture (DYABTlip), followed by Klett units and viable count measurement. At 30°C R3-1 showed normal rod shape at early and middle log phase (Fig. 4a). The rods became shorter at about the end of the log phase (Fig. 4b), and then most of the cells became short rods or oval shaped during stationary phase (Fig. 4c). The cells were small and homogeneous in size, distinctly different from the spherical shape and heterogeneous size seen at 42°C (Fig. 4h). When the temperature of the log-phase culture at 30°C (Fig. 4a) was shifted up to 42°C, the cells gradually became spherical (Fig. 4d, e, f, g, h); first they became shorter rods (Fig. 4d), then spherical with homogeneous size (Fig. 4e), and finally spherical with a heterogeneous size distribution (Fig. 4f to h). An hour after the culture was

shifted back to 30°C, after 90 min at 42°C, the spherical cells (Fig. 4e) were enlarged (Fig. 4i). In addition, the optical density decreased to  $\frac{1}{3}$  within 24 min after shift-down, and the viable count decreased to  $\frac{1}{2}$  of that expected from the value of 30°C at the time of shift-down (Fig. 3), indicating some cell lysis. Two hours after shift-down the cells appeared as large ovoid cells or large irregularly shaped cells (Fig. 4j). These morphological forms dominated the population for a while (Fig. 4j, k); then a few normal short rod cells appeared (Fig. 4l) and gradually became the predominant morphological form (Fig. 4m, n). When this culture entered stationary phase the cells were short rods or oval shaped (Fig. 4o), similar to the stationary-phase cells at 30°C (Fig. 4c). A few lysed cells were still visible. They were probably cells which had lysed upon initial shift down.

**Observations by scanning electron microscopy.** At 30°C most cells appeared as normal rods during log phase (Fig. 5a) with a length of 2 to 3  $\mu\text{m}$ . When the temperature was shifted up to 42°C the cells became shorter (Fig. 5b), and after 90 min at 42°C they became spherical

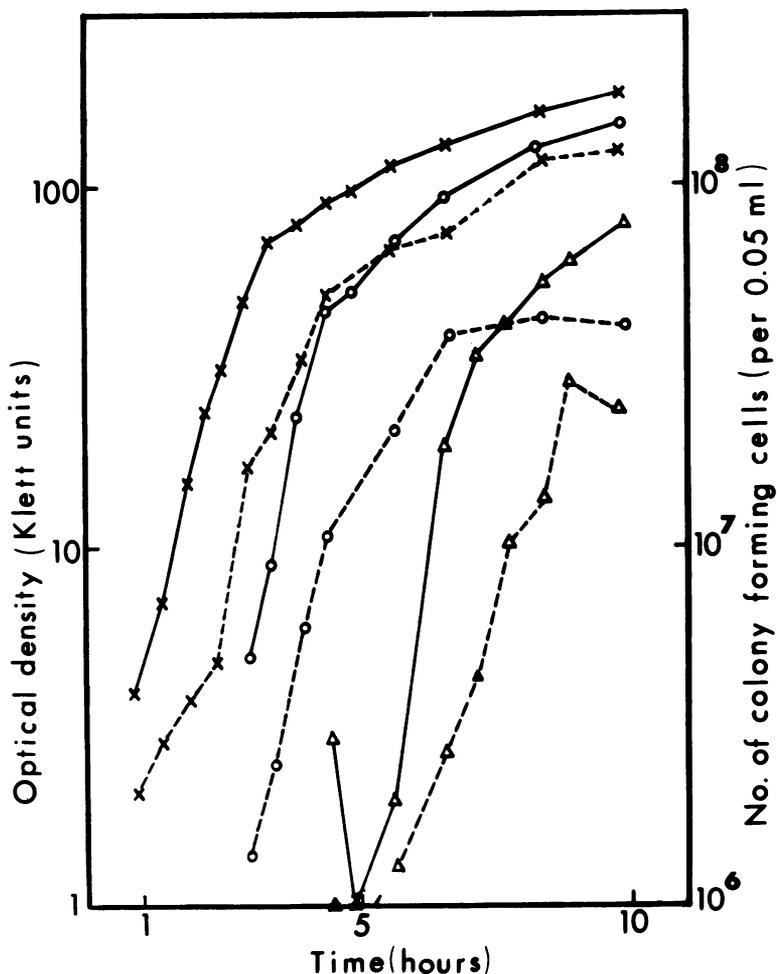


FIG. 3. Growth of strain R3-1 at 30 and 42°C. Growth of cultures at 30°C and temperature shift-up to 42°C or shift-down to 30°C were done according to the procedures in the text. The original culture at 30°C was started from a cell culture at midlog growth phase. For viable count, 0.1 ml of culture was withdrawn at various times and plated on DYABT plates after appropriate dilutions. After 1 day at 30°C the number of colonies was counted. Symbols: (x—x) optical density at 30°C; (o—o) optical density at 42°C after shift-up at 3 h; (Δ—Δ) optical density at 30°C after shift-down from 42°C at 4.5 h; (x----x) viable count at 30°C; (o----o) viable count at 42°C after shift-up; (Δ----Δ) viable count at 30°C after shift-down from 42°C.

(Fig. 5c). A constriction in the spherical cell appeared only on one side of the cell at about the middle. A few cells had a constriction near one end of the cell. These spherical cells showed prominent wrinkles in their envelope structure (Fig. 5c and d), which were not seen on rod-shaped cells at comparable magnification (data not shown). When the temperature was shifted down from 42 to 30°C, the spherical cells (Fig. 5d) became irregular rods (Fig. 5e), and then finally became very short rods or oval shaped (Fig. 5f), similar to cells grown to stationary phase at 30°C (Fig. 5g). Some of the monster cells (Fig. 5e) appear to have oversized envelopes.

**Observations by transmission electron microscopy.** R3-1 had a well-defined, condensed chromosome during log phase at 30°C (Fig. 6a). When the temperature was raised to 42°C, the chromosome became dispersed (25) and distributed to the cell periphery (Fig. 6b and c). After 90 min at 42°C most cells possessed dispersed chromosomes (Fig. 6d). The chromosomes returned to the condensed state gradually (Fig. 6e) after the temperature was shifted down to 30°C. Even at 30°C stationary-phase cells showed less condensed chromosomes (Fig. 6f) than did log-phase cells. The wild-type strain, R3-5, had fairly condensed chromosomes at 42°C (Fig. 6g), although less condensed than R3-

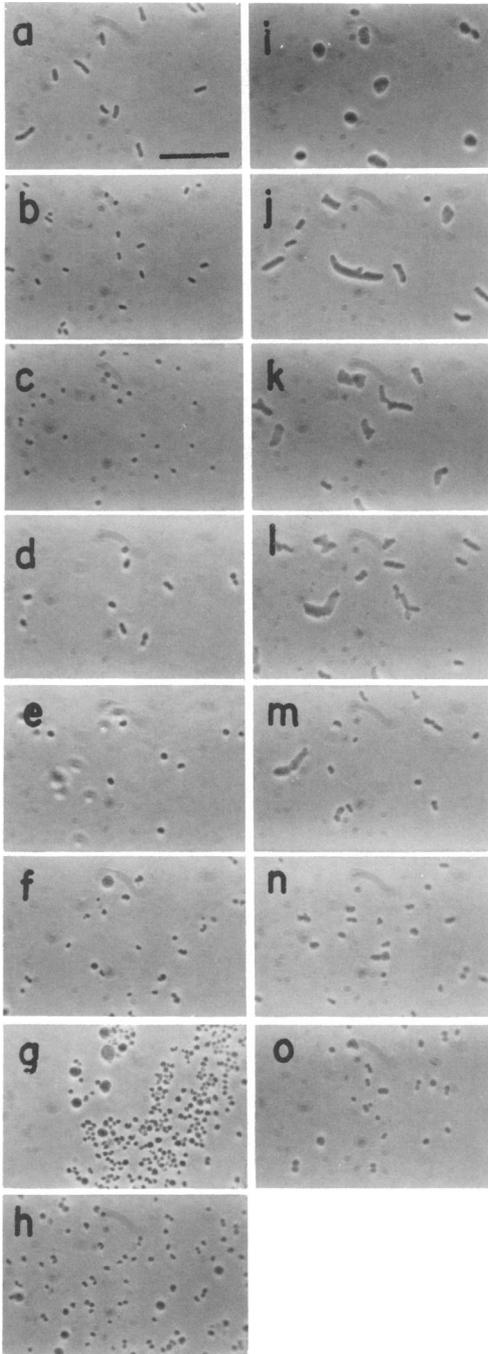


FIG. 4. Phase-contrast photomicrographs of strain R3-1 at 30 and 42°C. Samples of 3 ml of bacterial culture were withdrawn at various times in Fig. 3 and fixed in glutaraldehyde as described in the text. (a) 30°C culture just before temperature shift-up; (b) 30°C culture at the end of log growth phase (90 Klett units); (c) 30°C culture at the stationary growth phase (180 Klett units); (d to h) 55, 85, 220, 320, and

1 at 30°C (Fig. 6a).

R3-1 was grown at 42°C from a single colony, and the state of the chromosome in log- and stationary-phase cells was examined. Figures 6h and i show dispersed chromosomes in log-phase and stationary cells.

We examined the chromosomes in other spherical *E. coli* strains and found that all spherical strains examined showed considerable dispersion of their chromosomes. Figure 6j shows strain HL103, which is round shaped and has a morphological mutation (*rodA* or *rodX*) near *lip* (12). This particular picture shows a cell with a membranous structure inside a vesicle in the cytoplasm (6, 10). Figure 6k shows strain AR6, in late log growth phase, which is also round shaped and has a morphological mutation (*envB* or *rodY*) near *aroE* (12).

The high-magnification pictures of R3-1 grown at 42 and 30°C (Fig. 6l and m) showed little difference in their envelope structures (10).

**Cell number versus viable count.** Like other spherical mutants, strain R3-1 can form colonies on plates and can divide indefinitely in liquid culture at 42°C, although some round-shaped mutants grow slower than wild type. It is possible that a proportion of these round-shaped cells are dead and cannot divide. We examined this possibility by comparing the cell number (counted in a Petroff-Hausser chamber) and the viable count (colonies formed on plates) of log growth-phase cultures of strain R3-1 growing at 30 and 42°C. The cell number of strain R3-1 was about twice the viable count at both temperatures. We observed a similar discrepancy for strain AT1325lip9 (the recipient strain for transduction when R3-1 was constructed) at 42°C, but at 30°C the cell number and the viable count agreed well (data not shown).

**Asymmetric cell division on agar.** Figure 7 shows time-lapse photographs of cell division on agar medium at 30°C. These photographs are of strain CS1 (mecillinam resistant and *envB* or *rodY*). Identical observations have been made for strain R3-1, grown at 42°C on agar, and for strain HL103 (*rodA* or *rodX*), grown at 30°C on agar. Figure 7b shows the beginning of a constriction in two cells at the center of the picture. This constriction was asymmetrical (Fig. 7b and c) and was completed in 20 min (Fig. 7d). During the next 10 min a second constriction started (Fig. 7e) that was also asymmetrical and was completed in 15 to 20 min (Fig. 7f). One of the cells seemed to be lysed (Fig. 7g, arrow A), and

410 min, respectively, after temperature shift-up to 42°C; (i to o) 65, 130, 160, 195, 230, 265, and 320 min, respectively, after temperature shift-down to 30 from 42°C.

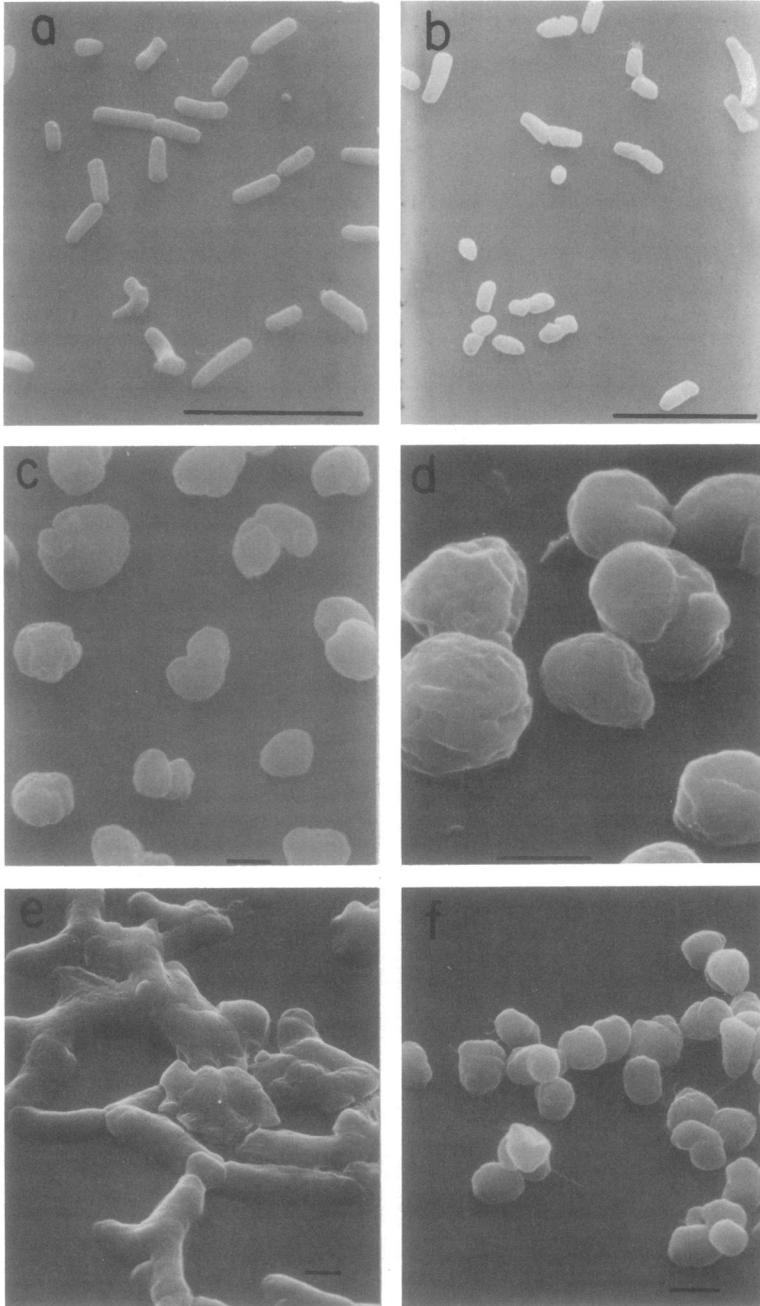


FIG. 5. Scanning electron micrograph of strain R3-1 during temperature shift-up and shift-down. (a) Midlog growth phase at 30°C; (b and c) 40 and 90 min, respectively, after temperature shift-up to 42°C; (d to f) 90, 160, and 400 min, respectively, after temperature shift-down to 30 from 42°C; (g) stationary growth phase at 30°C. Bar represents 10  $\mu\text{m}$  (a, b) or 1  $\mu\text{m}$  (c to g).

another divided earlier than its sister cell (Fig. 7g, arrow B). Cells divided in a plane perpendicular to the previous division plane (Fig. 7b to f).

#### Temperature shift-down of strain R3-1

from 42 to 30°C on agar. When a liquid culture of R3-1 was shifted down from 42 to 30°C, many cells with abnormal morphology appeared (Fig. 4). However, this was not the case if cells

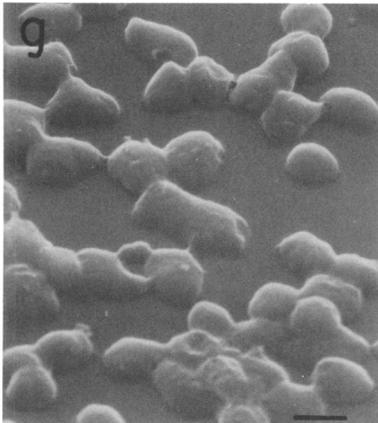


FIG. 5—Continued.

were placed on a solid medium at 30°C after growth in liquid at 42°C (Fig. 8). After the first division at 30°C cells were ovoid (Fig. 8c), and after the second division most cells were rod shaped (Fig. 8f). Only a few cells were abnormal in shape (arrow, Fig. 8g). This abnormal cell produced a normal rod cell at the next cell division (Fig. 8h and i).

**Effect of chloramphenicol and nalidixic acid on the morphological change of strain R3-1.** A culture of R3-1 was shifted up to 42°C from 30°C, and either chloramphenicol or nalidixic acid was added at the time of shift-up. Chloramphenicol at 100 µg/ml completely inhibited cell growth (measured by optical density), and there were no morphological changes in R3-1 after temperature shift-up in the presence of chloramphenicol. Nalidixic acid at 5 µg/ml inhibited cell growth by only 50% over 4 h; optical density in the absence of nalidixic acid reached 135 Klett units, whereas it reached 58 in the presence of nalidixic acid. The control culture showed round or ovoid cells, and the culture with nalidixic acid showed round cells or round cells with two short "ears." The effect of nalidixic acid on the morphological change of R3-1 is in contrast with its effect on the morphological change induced by mecillinam (21).

## DISCUSSION

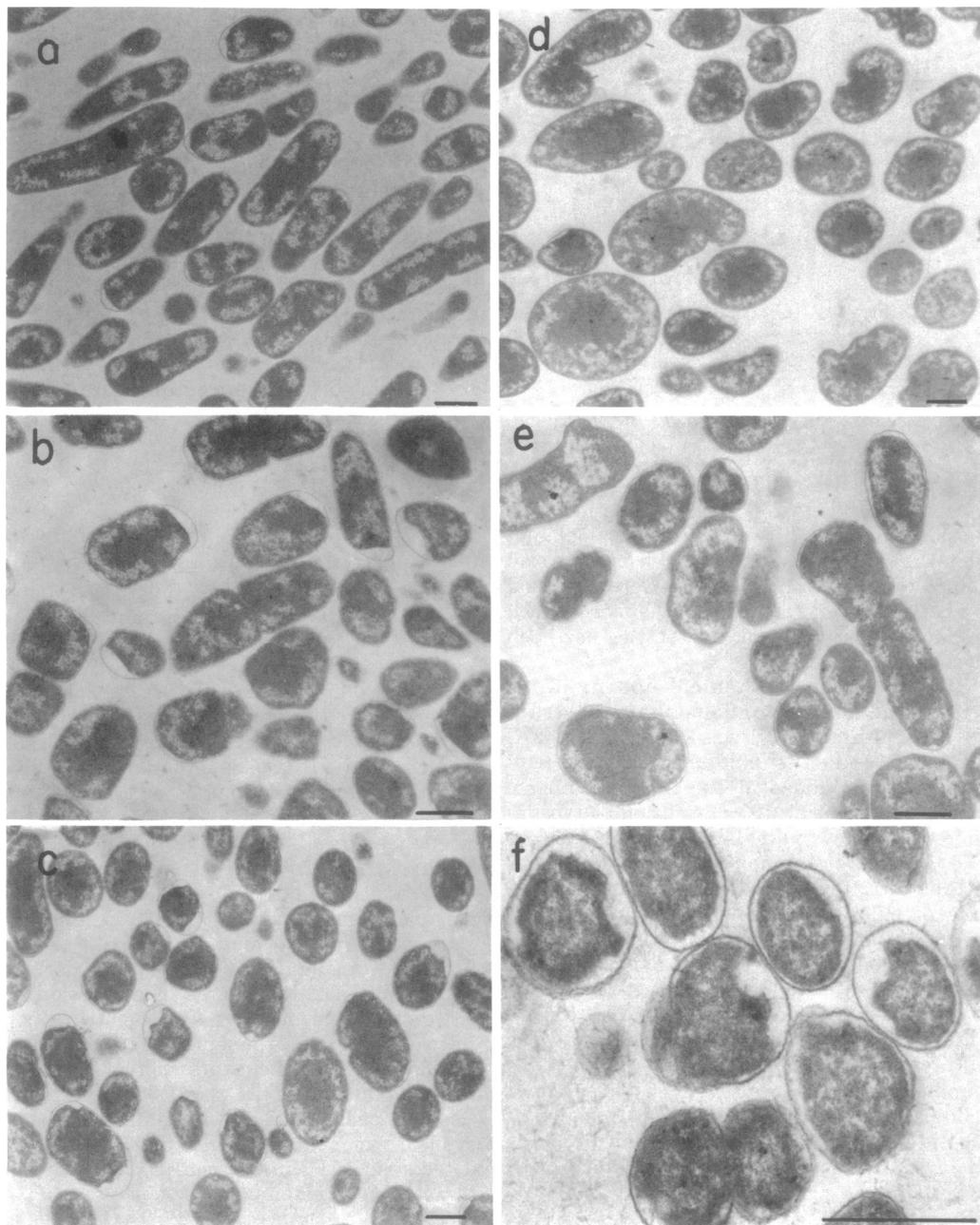
**Relationship between cell morphology, mecillinam resistance, and PBPs.** Four loci resulting in round morphology (*rod* genes) have been reported in *E. coli*: *rodA* (or *rodX*; 12, 22), *envB* (or *rodY*; 12, 33), *cya*, and *crp* (17). Mutants in *rodX* and *rodY*, which are similar to or probably the same as *rodA* and *envB*, respectively, were isolated with high frequency among mecillinam-resistant *E. coli* after NTG treatment (21 and this paper). We have previously

examined the relationship between mecillinam resistance (*mec<sup>r</sup>*) and round morphology (*rodX* and *rodY*) and showed that *rodX* and *mec<sup>r</sup>* are very closely linked or identical genes (12).

At 30°C the *rod(Ts)* transductant R3-1 was rod shaped (Fig. 4) and normally mecillinam sensitive both in liquid medium (Fig. 1b) and on plates (data not shown). At 42°C, it grew as spheres (Fig. 4) and was totally mecillinam resistant both in liquid medium (Fig. 1b) and on plates (data not shown). The recipient for transduction was also markedly more resistant to mecillinam at 42°C, but was distinctly more sensitive to higher concentrations (10 µg/ml) than was R3-1 (Fig. 1a). Thus R3-1, besides its *rod(Ts)* phenotype, is also temperature sensitive for mecillinam resistance. Both characteristics are mapped at about 14 min (Table 2) by their high degree of cotransduction with *lip* and must be the same as or closely linked to *rodX* (or *rodA*).

Transductant R3-1 and its parent R3 had detectable but reduced amounts of PBP2 at 30°C and lacked it at 42°C (Fig. 2 and unpublished data). The recipient strain AT1325lip9 and *lip<sup>+</sup> rod<sup>+</sup>* transductants such as R3-5 had normal amounts of PBP2 (Fig. 2) at both temperatures. The fact that PBP2 was missing in R3 and R3-1 at 42°C but present at 30°C is consistent with identity of the *rodX* (or *rodA*), *rod(Ts)*, and PBP2 genes. Moreover, the thermolability of penicillin binding to PBP2 in membranes of R3-1 grown at 30°C (unpublished data) strongly suggests that *rod(Ts)* lies in the structural gene for PBP2. A thermolabile PBP2 mutant was also found by Spratt (32; B. G. Spratt, personal communication). At 30°C, the *rodX* gene in the *rod(Ts)* mutant was expressed sufficiently to maintain the rod shape in early log phase, although the degree of expression was less than normal and was insufficient to maintain rod shape in postexponential growth in liquid media. At 42°C the expression of *rodX* was further reduced in the *rod(Ts)* mutants, such that the cells could not maintain a rod shape even in exponential growth, and there was no detectable PBP2 in the cell. It seems possible that *rod(Ts)* and temperature sensitivity of both mecillinam resistance and PBP2 expression are all due to a single lesion in the *rodX* (or *rodA*) gene, since it seems unlikely that three different closely linked mutations would simultaneously be temperature sensitive. However, one should be careful to reach this conclusion, since NTG induces multiple mutations in a small region (8).

At 30°C, PBP2 is present in normal *E. coli* strains in about 10 to 20 copies per cell (31). In the *rod(Ts)* mutants, this was reduced about eightfold (Fig. 2a), giving only one to three mol-



**FIG. 6.** Transmission electron micrograph of various strains during temperature shift-up and shift-down. (a to f) Strain R3-1: (a) 30°C at midlog growth phase; (b to d) 20, 60, and 90 min, respectively, after temperature shift-up to 42°C; (e and f) 150 min and 10 h, respectively, after temperature shift-down to 30 from 42°C. (g) Strain R3-5 in midexponential growth phase at 42°C; (h) strain R3-1 in midexponential growth phase at 42°C; (i) strain R3-1 in stationary phase at 42°C; (j) strain HL103 in late exponential growth phase at 30°C; (k) strain AR6 in late exponential growth phase at 30°C; (l) strain R3-1 at 42°C; (m) strain R3-1 at 30°C. Bar represents 1  $\mu\text{m}$  (a to k) or 0.1  $\mu\text{m}$  (l and m).

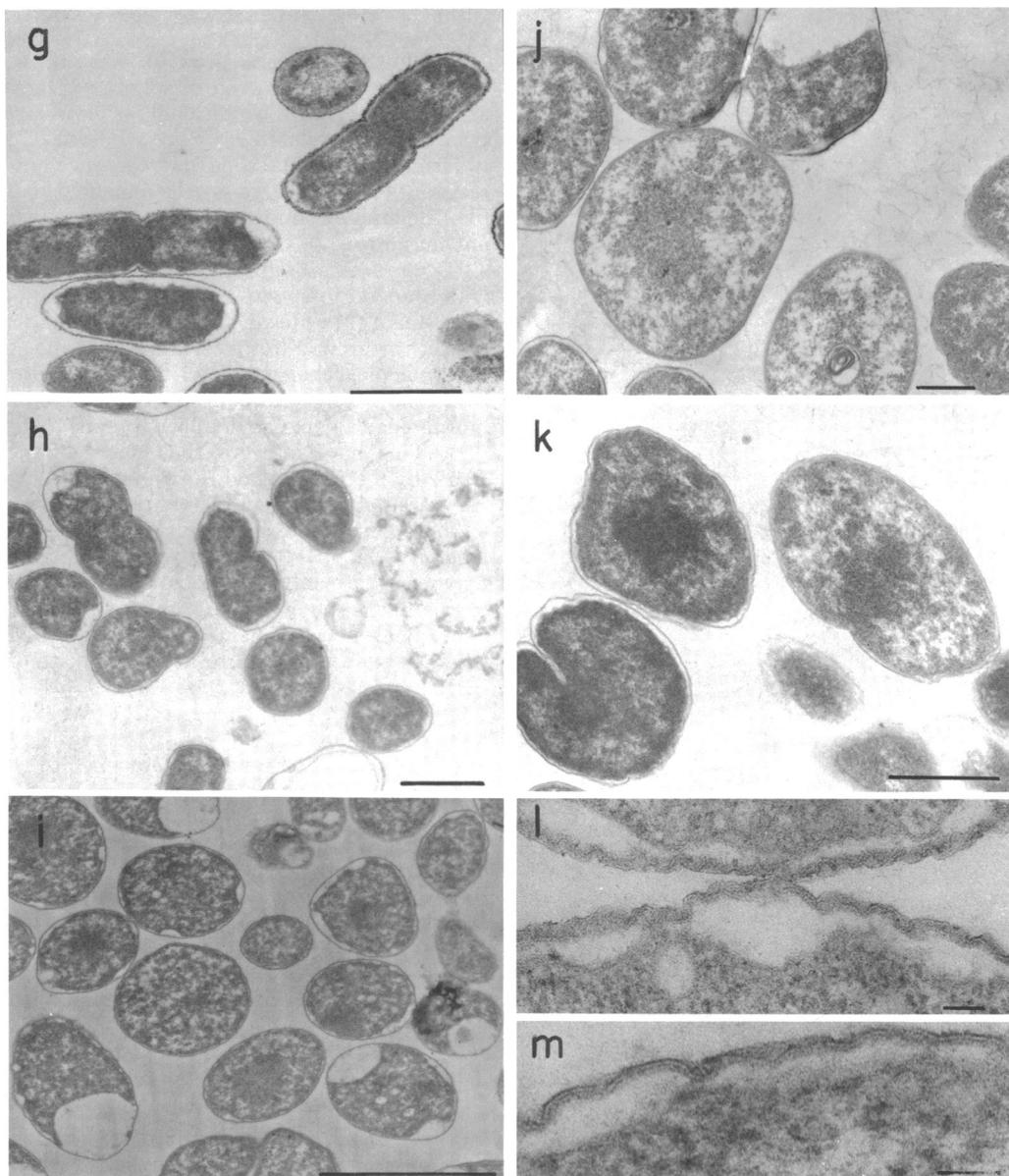


FIG. 6—Continued.

ecules of PBP2 per cell, apparently enough to maintain rod shape. It is also possible that normal amounts of a modified PBP2 are produced and that this modified molecule has reduced affinity for benzyl penicillin and is temperature labile.

**Morphological change from rod to round.** Strain R3-1 produced normal rod-shaped cells when growing exponentially at 30°C. After shift of the cultures to 42°C, turbidity and viable

counts continued to increase exponentially (Fig. 3), but cellular morphogenesis and cell division control became altered so that, at the subsequent cell division, an ovoid cell was produced (Fig. 4 and 5). If rod-to-sphere conversion is entirely dependent on loss of PBP2 function and if the function is limited to a certain phase of the cell cycle (e.g., septation), then initiation of this conversion process would be dependent on the completion of septation events already in

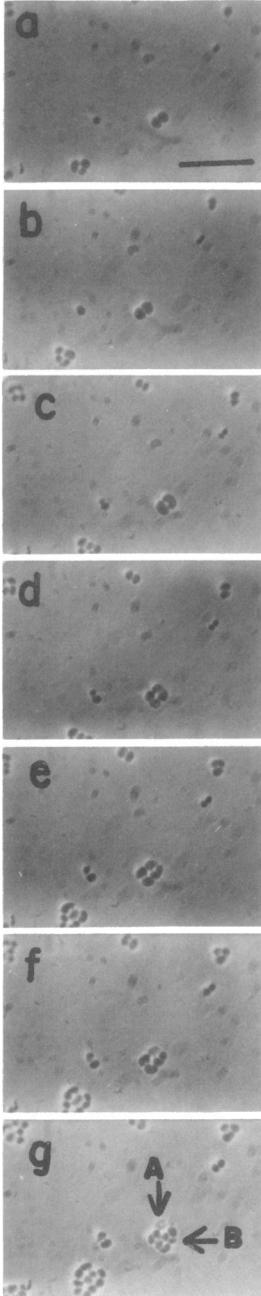


FIG. 7. Time-lapse photomicrograph of strain CS1. A liquid culture of strain CS1 at midlog phase at 37°C was placed on agar and observed by phase-contrast microscopy at 37°C, as described in the text. (a to g) Pictures were taken at 0, 12, 23, 32, 42, 50, and 61 min, respectively.

progress in the rod-shaped cells. Asynchrony in rod-to-sphere conversion would be expected, as observed (Fig. 5b), although the conversion of

the entire population occurs smoothly within two generations.

Mecillinam interacts directly with PBP2 in sensitive cells, causing rod-to-sphere conversion and cell death. The conditional lesion in strain R3-1 is clearly related since growth at 42°C led to formation of round cells that were mecillinam resistant (Fig. 1), but these cells remained viable, like the stable round-shaped mecillinam-resistant mutants (12). Conversion in strain R3-1 at 42°C requires protein synthesis, as is the case with the rod-to-sphere conversion induced by mecillinam (21). However, nalidixic acid did not inhibit the growth (for a certain period) nor the morphological change of R3-1. This is in contrast to the inhibitory effect of nalidixic acid on the morphological change induced by mecillinam (21). This may indicate that inactivation of PBP2 by mecillinam and the mutation in strain R3-1 are not equivalent. The difference may be either quantitative, such that mecillinam may inactivate all PBP2, whereas high temperature may inactivate only a portion of mutated PBP2, or qualitative, such that mecillinam may inactivate some cell components in addition to PBP2, whereas high temperature may inactivate only PBP2.

The round cells produced at 42°C were originally small, but after several division cycles the size of round cells became heterogeneous. This heterogeneity in size is generally seen for round cells of *rodA* (or *rodX*) and *envB* (or *rodY*) mutants (12; unpublished data), as for round cells induced by mecillinam (21). The larger-than-average cells may be cells about to divide, or moribund cells with damaged envelopes that are inflated due to osmotic pressure. The larger round cells tended to puncture readily on agar during growth and division (Fig. 7g; unpublished data on HL103), although both larger and smaller cells can divide on agar (unpublished data). The chemistry of the round cell envelope has not been determined. Henning et al. (10) isolated round "murein sacculi" from a round mutant similar to R3-1 by sodium dodecyl sulfate extraction. It thus seems likely that rod-to-sphere conversion requires rearrangement of the peptidoglycan matrix and is not simply an osmotic effect.

**Morphological change from round to rod.** The round cells of strain R3-1 grew well at 42°C in either liquid or solid media (Fig. 3). The morphological changes after temperature shift-down were drastic; the process of emergence of normal rod cells from abnormal cells is complex, and at least two interpretations exist. Most of the cells may become abnormal in shape, and some of these may subsequently produce normal rods after cell division, while the rest lyse. Alter-

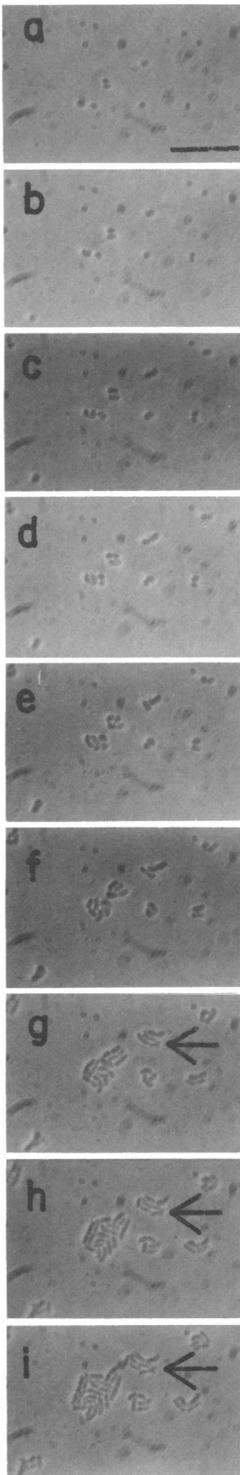


FIG. 8. Time-lapse photomicrograph of strain R3-1 after temperature shift-down from 42 to 30°C. Liquid culture of strain R3-1 at midlog phase at 42°C

natively, some of the round cells, perhaps those that are smaller and younger, may convert themselves into rods, while the rest become abnormal and eventually lyse. Both possibilities may occur to various extents depending on conditions. Figure 8g to i shows that a triangular cell produces a normal rod cell on agar, and almost all originally round cells produce normal rods after one to two rounds of cell division on agar (Fig. 8), in contrast to the aberrant morphological changes in liquid medium (Fig. 4). The branching cell with a loose envelope seen in the micrograph in Fig. 5e is typical of monster cells seen in liquid medium.

#### Multiple characteristics of round cells.

The round cells of strain R3-1 produced at 42°C (Fig. 5c and d) were much more wrinkled than the rod-shaped cells produced at 30°C. Similar wrinkles were seen in the round cells of *envB* mutants (unpublished data) and on normal cells treated with mecillinam (7). They may result from osmotic damage to these fragile cells, or possibly from attempts by the cell to maintain an envelope-to-mass ratio suitable for rod-shaped cells.

The dispersed chromosomes of these cells contrast with the condensed chromosomes of normal *E. coli* cells, fixed by the Kellenberger technique. This condensed state seems to depend on the concentration of cations in the nucleoplasm, since the condensed chromosome disperses under different treatments for pre-fixation (4, 34, 35). Since condensed nuclear areas can be observed in live, dividing cells in gelatin by phase-contrast microscopy (19), and a highly folded DNA can be isolated physically (36), the chromosome of wild-type *E. coli* in vivo is probably relatively condensed. The nuclear dispersion seen in R3-1 at 42°C (Fig. 6c and d) has been observed in *rodA* (or *rodX*) and *envB* (or *rodY*) mutants (Fig. 6j and k; 3, 21, 22, 25) and in wild-type *E. coli* that was induced to become round by mecillinam (21). Since round cells seem to have altered, osmotically fragile envelopes (this section; 21), round cells may have a lower concentration of cations inside the cell or may be more easily damaged during sample preparation for electron microscopy. This nuclear dispersal seems to be consistent with viability.

The constriction of round-cell division starts at about the cell equator (Fig. 7) and is usually asymmetrical (Fig. 5 for *rodA* and Fig. 7 for *envB* mutants). Similar asymmetrical constrict-

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was placed on agar at 30°C and observed under phase-contrast microscope at 30°C. (a to i) Pictures were taken at 0, 36, 68, 87, 104, 123, 146, 164, and 178 min, respectively, after the transfer of strain R3-1 onto agar at 30°C. Arrow indicates an abnormal cell.

tion was observed for an *envB* mutant (3, 25) and a wild-type cell treated with mecillinam (21, 23). In contrast to our observation, Matsuzawa et al. (22) reported normal symmetrical septum formation for a *rodA* mutant. Even though the constriction starts asymmetrically, the two daughter cells look like the products of symmetrical division at or near the end of the process (compare Fig. 7a, d, and c), so the division phase of cells is critical for determining morphology. Alternatively, a few round cells may divide symmetrically while others divide asymmetrically (23). Micrococci and staphylococci typically divide in planes perpendicular to that involved in the previous cell division, producing regular packets if cytokinesis is symmetrical and cells remain attached (20). Whereas the streptococcal mode of cell division in parallel planes can readily be rationalized (5) on the basis of laterally symmetrical wall growth zones, models for the micrococcal model are less satisfactory. The Jacob model for bacterial nuclear segregation (14, 27, 28), based on permanent attachment to the envelope and controlled envelope extension, is not easily adapted to this division model, which also seems to be true for the round cells of strain R3-1 (Fig. 7). If, however, totally asymmetric septation is assumed, as may occur in strain R3-1 at 42°C, then the model of Jacob et al. (14) may be invoked to explain the division pattern observed in agar (see Fig. 9). Chromosomes are segregated (Fig. 9b) by the enlargement of envelope material between daughter chromosomes (14). This enlargement is symmetrical, as is assumed for rod cells. Initiation of septation starts by constriction between the two daughter chromosomes (Fig. 9c). At the end of asymmetric constriction (Fig. 9d) two cells separate. Since free movement of cells is restricted by solid agar, the two cells end up with the two daughter chromosomes facing each other (Fig. 9e). These two cells will repeat the above process. Thus, the next constriction sites will be facing each other, and this makes the next division plane perpendicular to the previous division plane (Fig. 9d to f).

Since the process from Fig. 9d to Fig. 9e requires the inhibition of free movement of cells by solid agar, the perpendicular planes of successive division should not be seen in liquid medium, such as methocel (18).

If the assumption is correct that the asymmetric constriction initiates in the middle of two daughter chromosomes, the fact that the constriction usually initiates on the particular side of the envelope of each cell that is in contact with the other (Fig. 7c and e) might support the

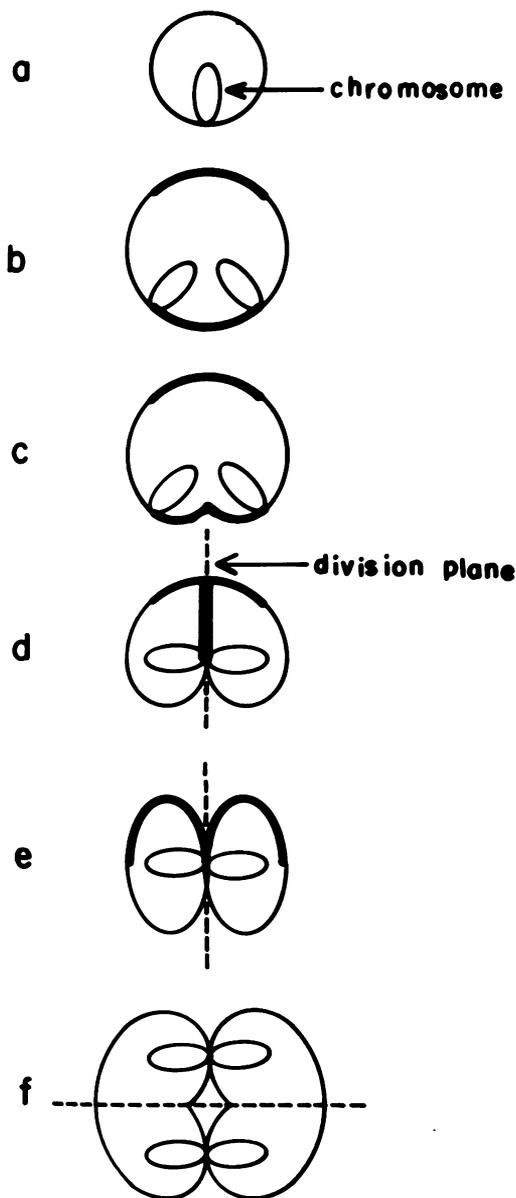


FIG. 9. Model of asymmetrical cell division on agar; (a to e) show the whole process of a cell division. The next cycle of cell division is shown in (f). See the text for details. (a) Single-size cell; chromosome is represented by a small oval shape. (b) Double-size cell; thick line represents enlargement of envelope (this doesn't necessarily mean a freshly synthesized envelope). (c) Initiation of asymmetric constriction. (d) End of asymmetric constriction; division plane is represented by a dotted line. (e) Cell separation on agar; friction between cell surface and agar keeps the daughter cells from moving away. (f) Initiation of the next division.

idea that chromosomes attach to membrane or that they are somehow fixed at particular sites in a cell.

Melchior et al. (23) observed that daughter nuclei tended to separate in a plane perpendicular to the equatorial plane of septation of the parent cell in mecillinam-treated round cells. Adler et al. (2) reported that cell division may occur in a variety of planes, and no obvious pattern was observed.

The genetic evidence suggests that all of the phenotypes of strain R3-1 are due to a single mutation (or to very closely linked mutations) in the *rodA* locus that causes all of the temperature-dependent changes in morphology, mode of division, PBP2 content, and mecillinam resistance (12; unpublished data). Mutants in *rodY* (*envB*) have similar morphology though they do not lack PBP2 (unpublished data). Perhaps the gene products of *rodA* (PBP2) and *rodY* are both required for maintenance of normal rod cell shape. Genetic and biochemical study of the gene product of *rodA* and its interaction with other components of the cell envelope may lead to a better understanding of the process of septation, which is an important aspect of cell division.

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